Roles of the Polar Cytoplasmic Region in Meiotic Divisions in Oocytes of the Sea Cucumber, *Holothuria leucospilota*

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Abstract. The sea cucumber oocyte has a marked cytoplasmic protrusion at its presumptive animal pole. The role of this cytoplasmic region (called the "pole" in this paper) in meiotic divisions was investigated. With maturation, the germinal vesicle (GV) migrates to the pole and breaks down. When the migration of the GV was impeded by compression, a pair of asters developed in the cytoplasmic region at the pole. A meiotic spindle formed when these two asters united with the nucleoplasmic area after breakdown of the GV. The origin of these asters was then examined by transecting oocytes microsurgically. Upon maturation, the fragments containing the pole, but lacking the GV, developed a pair of asters in the cytoplasmic region at the pole. Fragments containing both the pole and the GV formed a meiotic spindle. However, no asters formed in fragments lacking the pole (either containing or lacking the GV). The results demonstrate that the pair of asters are the organizing centers of the spindle, and that they are derived from the pole, indicating that the centrosome(s) resides in the pole of the oocyte.

Introduction

Prophase-arrested oocytes of sea cucumbers have a conspicuous cytoplasmic protrusion (Gerould, 1896; Ohshima, 1921; Inaba, 1930; Maruyama, 1980; Smiley and Cloney, 1985). With maturation, polar bodies form by a pinching-off of the protrusion (Maruyama, 1980, 1981, 1985). Thus, the protrusion is a marker of the presumptive animal pole. Similar protrusions have been described in oocytes of sea lilies (Holland *et al.*, 1975) and of some sea urchins (Jenkinson, 1911; Lindahl, 1932; Monné, 1946).

The protrusion in sea cucumber oocytes contains fibrillae (Ohshima, 1921, 1925; Inaba, 1930), and in the growing oocytes of *Holothuria monacaria*, the fibrillar structure is reported to originate from a hematoxylin-stained oval body (Oka, 1940). Electron microscopy revealed that an array of microtubules extend from the protrusion to the GV in oocytes of another sea cucumber, *Stichopus californicus* (Smiley and Cloney, 1985). In starfish oocytes, a pair of asters and centrosomes (called "premeiotic asters") are pre-existing and associated with the cell surface at the presumptive animal pole (Wilson and Mathews, 1895; Schroeder and Otto, 1984; Schroeder. 1985a, b; Picard *et al.*, 1988).

The present study was initiated to elucidate the role of the cytoplasmic region of the protrusion in meiotic divisions in oocytes of the sea cucumber, *Holothuria leucospilota*. I found that a pair of asters responsible for meiotic spindle formation are derived from the protrusion of the prophase-arrested oocyte. The cytoplasmic region of the protrusion in oocytes will be referred to, in this paper, as the "pole"; it corresponds, in terms of classical embryology, to the presumptive animal pole.

Materials and Methods

Oocytes

Adult specimens of *H. leucospilota* were collected in the vicinity of the Seto Marine Biological Laboratory (Shirahama, Wakayama prefecture) from July through August. Ovaries and isolated oocytes were prepared as described previously (Maruyama, 1980, 1985). Oocytes within, or isolated from, the ovaries were immature, arrested at prophase-I.

Observation of maturation process

Oocytes in ovaries were induced to mature by treatment with radial nerve extracts, as described previously (Ma-
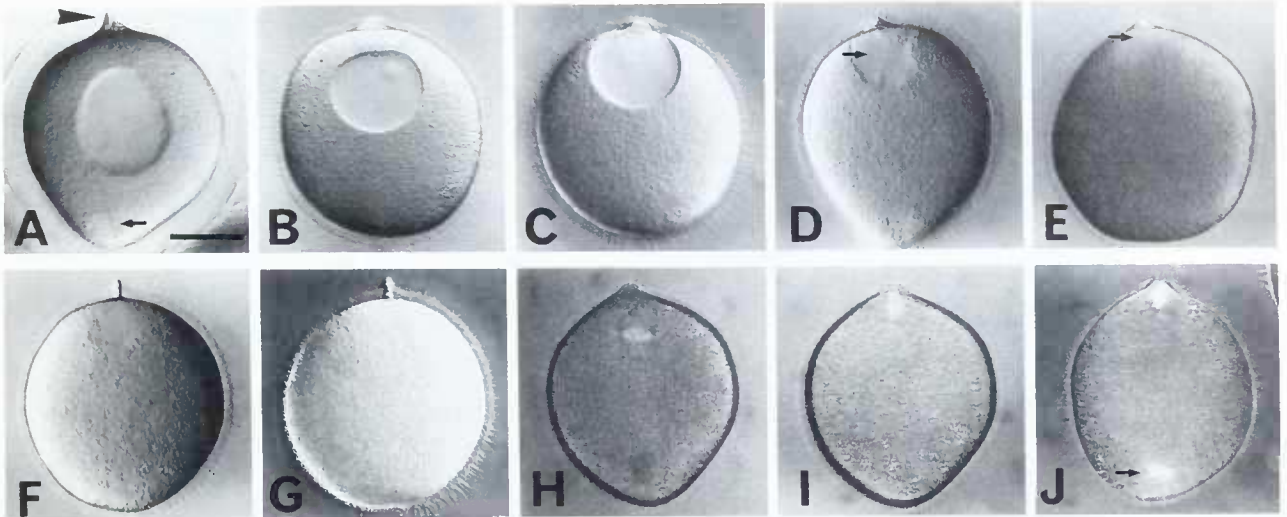
Figure 1. Maturation in intact oocytes of *Holothuria leucospilota* Oocytes were induced to mature by treatment with radial nerve extracts and were observed with Nomarski (A–G) and polarizing (H–J) microscopes. A: A prophase-arrested oocyte with follicle cells. A cytoplasmic protrusion (top, indicated by an arrow-head) and a cytoplasmic islet (bottom, indicated by an arrow) are seen. B: 14 min. Germinal vesicle (GV) migration. C: 17 min. D: 27 min. Breakdown of GV. The arrow (also in E) indicates chromosomes. E: 50 min. Metaphase. F: 65 min. Telophase. The first polar body is forming from the elongated protrusion. G: 100 min. The second polar body is forming. H: A newly formed meiotic spindle in an oocyte at 30 min. I: The meiotic spindle at 36 min (the same oocyte as in H). The spindle attaches to the pole with one end. J: The meiotic spindle (top) at 57 min. The birefringent body (arrow at the bottom) is the clear spot. Temperature: 27–28°C. Bar in A: 50 μm (for A–J).

ruyama, 1985). Eight to ten minutes later, the oocytes were isolated, washed once, and used for observations. Maturation could also be induced by treating isolated oocytes with dithiothreitol (DTT: Wako Pure Chem. Comp. Ltd., Osaka) dissolved in seawater at a final concentration of 1 mM (Maruyama, 1980). At appropriate intervals, maturing oocytes were pipetted onto a glass slide and covered with a cover slip. A pair of glass rod spacers (about 200 μm thick), placed between the cover slip and the glass slide, permitted the observation of intact oocytes without compression. The specimens were observed with a light microscope through Nomarski or polarization optics.

In this paper, "developmental time" is reckoned in minutes from the start of induction of maturation. Manipulations and observations were made at room temperature (24–30°C).

### Compression of oocytes to suppress migration of germinal vesicles

Isolated oocytes were induced to mature by treatment with seawater containing 1 mM DTT. Five to twenty minutes after the start of induction, a small number of oocytes at GV-containing stages were transferred to a drop of seawater containing 1 mM DTT on a glass slide; the oocytes were then compressed under a fragment of cover slip, spaced by three pieces of glass rod (50–70 μm thick).

Once the drop of seawater under the cover slip had been sealed with silicone oil (SH-1107, Nakarai Chemicals Ltd., Kyoto), the specimen could be observed successively through Nomarski optics. The oocytes examined were those in which the main axis (defined by the pole) was parallel to the glass surfaces. For each oocyte, the "distance" between the pole and the nucleus—defined by the shortest length from the pole to the GV, either just before or after its breakdown—was measured with an ocular scale calibrated with a stage micrometer. The largest diameters of some oocytes and their GVs were measured. When the oocytes were under compression, the cell and GV diameters were, respectively, about 1.4–1.5 times and 1.1–1.2 times larger than those of uncompressed oocytes.

Microsurgical operation of oocytes

Isolated oocytes were transected into two fragments, under a dissecting microscope, with a glass microneedle (*cf.* Maruyama *et al.*, 1986). The transections were made at various locations with respect to the pole, as depicted in Figure 4. The fragments resulting from this surgery were treated with seawater containing 1 mM DTT to induce maturation. Five to twenty minutes later, they were transferred onto glass slides and observed as described above.
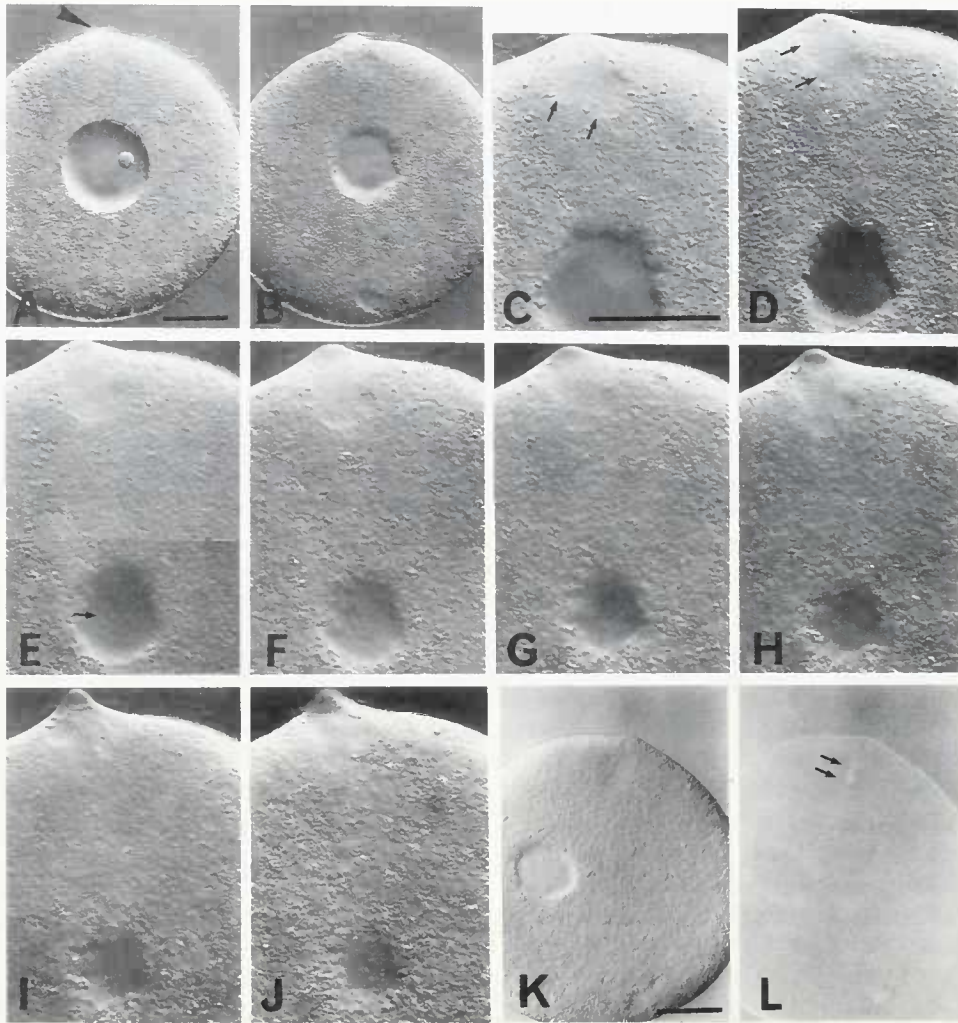
Figure 2. Dissociation of spindle organization-centers from the nucleus in oocytes compressed before GV migration (at 10 min of maturation). The oocytes were successively observed (A–J and K–L illustrate different oocytes). A: 23 min. The arrow-head points to the pole. B, C: 33 min. The GV has broken down. The distance between the GV and the pole is 72 μm. A pair of asters (indicated by arrows; also in D) at the pole (top), and the clear spot (bottom) at the antipole, are seen. D: 48 min. E: 58 min. The arrow points to the chromosomes. F: 63 min. G: 73 min. H: 83 min. I: 88 min. J: 98 min. K: Another oocyte at 40 min. A pair of asters are some distance from the nucleoplasmic area. L: The same oocyte as K, observed under a polarizing microscope. The pair of asters show a spindle-like figure. A birefringent body (bottom-right) is the clear spot. Bars: 50 μm (in A for A–B, in C for C–J, and in K for K–L). Temperature: 24–27°C.

Results

Meiotic events in intact oocytes

In prophase-arrested oocytes, the GV was located centrally or slightly off-center toward the pole (Fig. 1A). The GV began to migrate toward the pole 11–15 min after the start of maturation (27–29°C), and finally became associated with the pole (Figs. 1B, C). Follicle cells surrounding each oocyte were detached during the migration. The GV broke down at about 20 min (Fig. 1D), and the disap-

pearance of its membrane left a transparent nucleoplasmic area adjacent to the pole. Chromosomes and two small birefringent asters appeared there, and a spindle with two astral foci formed (Fig. 1H). Chromosomes were aligned to form a metaphase plate (Fig. 1E).

The newly formed meiotic spindle was oriented obliquely or vertically with regard to the main axis of the oocyte, defined by the pole (Fig. 1H). Through its rotation at 30–40 min (Fig. 1I), the spindle was "attached" to the pole with one end (Fig. 1J). With this attachment, cytoplasmic granules were displaced from the polar region
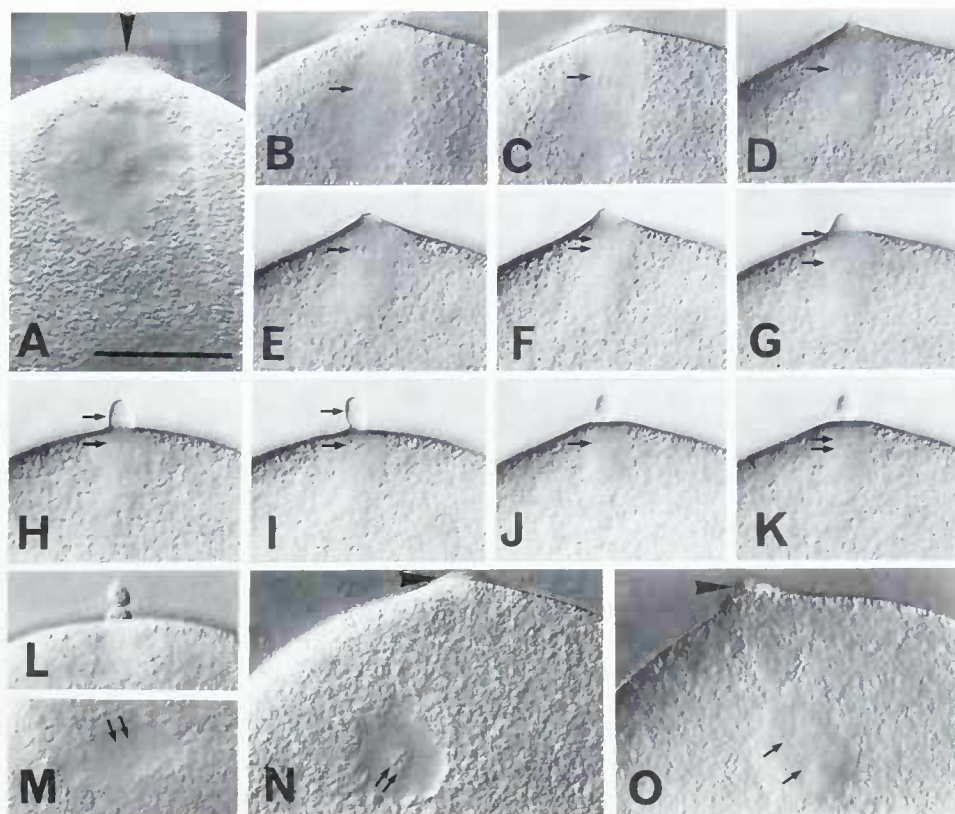
Figure 3. Meiotic spindle formation or polar body formation in an oocyte compressed after migration of the GV (at 20 min, A–L), and in oocytes compressed from the beginning of GV migration onward (at 15 min, M–O). Maturation was induced either with 1 mM DTT (A–L, M and O) or with radial nerve extracts (N). A: 25 min. The arrow-head points to the pole. B: 35 min. Metaphase. The arrow points to the chromosomes (also in C–K). C: 45 min. D: 55 min. E: 65 min. F: 70 min. Early anaphase. G: 74 min. Late anaphase. H: 75 min. I: 76 min. Telophase. The first polar body is forming. J: 119 min. Second metaphase. K: 123 min. Second anaphase. L: 128 min. The second polar body is forming. M: Anaphase chromosomes (arrows) in an oocyte at 70 min. The distance between the GV and the pole was 72 µm. N: Anaphase chromosomes (arrows) in an oocyte at 66 min. The distance between the GV and the pole was 32 µm. The arrow-head points to the pole (also in O). O: Telophase chromosomes (arrows) in an oocyte at 67 min. The distance between the GV and the pole was 36 µm. Temperature: 24–27°C. Bar in A: 50 µm (for A–O).

(Fig. 1E). Then the pole elongated radially, and one set of chromosomes passed through the base of the pole (Fig. 1F). The pole was constricted at its base to form the first polar body. The second polar body was formed just beneath the first (Fig. 1G, and also see Fig. 3I–L).

A transparent cytoplasmic islet ("clear spot") occurs in the cytoplasm of maturing oocytes at the side opposite that of the pole (cf. Fig. 2B; Maruyama, 1981). Observations with polarization optics revealed that the spot is birefringent (Fig. 1H, J). By careful observation, the spot could be traced back to the prophase-arrested oocytes (Fig. 1A, arrow).

Oocytes treated with radial nerve extracts and those treated with DTT matured similarly and had similar morphological features, as shown previously (Maruyama, 1980, 1985).

Dissociation of meiotic spindle-organizing centers from the nucleus

Maturing oocytes at GV-containing stages were subjected to compression under a cover slip to impede the migration of the GV. When oocytes at 5 or 10 min (i.e., before GV migration) were compressed, the GV broke down at an ectopic site apart from the pole (Fig. 2A, B), and the nucleoplasmic area thus formed remained there (Fig. 2). A pair of asters appeared at the pole just after GV breakdown (Fig. 2B, C); they showed only weak birefringence under a polarization microscope (Fig. 2L). At first, they moved away from the pole for a short distance, and then returned to the pole (Fig. 2D). As the asters moved to the pole, cytoplasmic granules contained in the polar region were displaced (Fig. 2D–J). Surprisingly,

Table I

Occurrence of meiotic spindles and polar bodies in oocytes under compression[1]

Initiation of compression	No. of oocytes	Distance[2]	Meiotic spindles[3]		Polar body[4] formation
			+	−	
Before migration	31 (9)	98 [70–148]	2 (1)	29 (8)	0 (0)
During migration	34 (13)	35 [12–110]	31 (12)	3 (1)	17 (6)
After migration	26 (9)	5 [0–28]	26 (9)	0 (0)	22 (8)

[1] Oocytes were induced to mature by treatment with 1 mM DTT or radial nerve extracts, compressed, and observed successively (24–27°C). Results from the latter were given in parentheses as fractions of cases.

[2] The "Distance" is the mean value with its range (brackets) in μm between the pole and the nucleus, measured for each oocyte at a time just before or after breakdown of the germinal vesicle.

[3] The meiotic spindle formation was examined through successive observations from about 30 min up to about 70 min. +, spindle formed; −, did not.

[4] Polar body formation was examined through further observations of each oocyte up to 96–190 min.

chromosomes appearing in the nucleoplasmic area remained there without forming karyokinetic figures (Fig. 2E–J). Thus, most (29/31) of the oocytes failed to form meiotic spindles (Table 1). Nevertheless, a small fraction (3/29) of these oocytes did form a metaphase-like chromosome configuration at a later time (at 120–140 min). In the remaining 2 out of 31 oocytes, metaphase to anaphase figures were detected at 50–70 or 55–80 min (Table 1).

Maturing oocytes at 11–16 min (*i.e.*, during GV migration) were then compressed (Table 1). The GV broke down near the pole (*cf.* Fig. 3N, O). A pair of asters were first detected, either at the margin of the nucleoplasmic area facing the pole, or in the cytoplasm adjacent to the pole. The asters then moved into the nucleoplasmic area, and chromosomes in the nucleoplasmic area were aligned to form a metaphase plate at 30–50 min. Most (31/34) of the oocytes formed meiotic spindles (Table 1).

When oocytes were compressed as late as 19–20 min (*i.e.*, after GV migration), the nucleoplasmic area formed at the pole (Fig. 3A), and in these cases, meiotic spindles formed (Fig. 3B–L, Table 1). In summary, meiotic spindle formation in compressed oocytes is apparently dependent upon the time of compression.

Polar bodies formed in oocytes under compression (Table 1). This occurred in cases where the metaphase

spindle moved, and "attached" to the pole (Fig. 3B–E). The first polar body formed by a pinching-off of the protrusion at its base (Figs. 3F–I), and the second polar body formed just beneath the first (Figs. 3J–L), as in uncompressed oocytes. In contrast, when the metaphase spindle failed to move and attach to the pole, polar bodies failed to form, but anaphase movement of chromosomes still occurred (Fig. 3M–O).

As shown in Table I, spindle and polar body formation failed, even in those compressed oocytes that had been obtained from ovaries stimulated with radial nerve extracts. Thus, such failures are not due to the effects of DTT used for maturation. The distance between the pole and the nucleus at the time of GV breakdown may be important for the successful formation of the meiotic spindle and polar bodies in oocytes under compression (Table 1).

The results show that a pair of asters develop in the cytoplasmic region at the pole after GV breakdown, and that the asters are required for organizing the meiotic spindle.

The pair of asters required for meiotic spindle formation are derived from the pole of the prophase-arrested oocyte

Prophase-arrested oocytes were microsurgically transected (Fig. 4), and the GV-enucleate fragments, whether containing the pole or not, were treated to induce maturation and observed (Table II).

Fragments containing the pole developed a pair of asters in the cytoplasmic region at the pole (Fig. 5A–D), whereas fragments lacking the pole did not. Two asters were invariably observed at 20–40 min (Table II); later (at 80 min or more), however, about half of the fragments had four asters. This shows that in fragments lacking the contents of the GV, each of the asters had split into two. Autonomous replication of centrosomes has been shown in starfish oocytes deprived of GV materials (Picard *et*
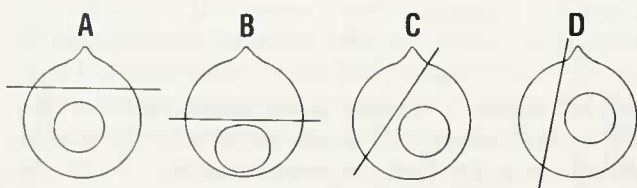
Figure 4. Transection of prophase-arrested oocytes. The oocytes were transected at one of the planes indicated (A–D) with reference to the protrusion marking the presumptive animal pole. On transection, the GV ends up in one of the daughter fragments.

al., 1988) and in enucleated sea urchin embryos (Lorch, 1952; Sluder *et al.,* 1986).

Fragments with a GV, but lacking the pole, were obtained and induced to mature (12 from the bisection B and 11 from the bisection C in Fig. 4). The GV still broke down, but the asters did not form. The chromosomes did not form karyokinetic figures and remained in a single cluster in the nucleoplasmic area (Fig. 5E). On the other hand, most (8 out of 9) fragments with both a GV and a pole region (produced by transecting prophase-arrested oocytes through plane D, Fig. 4) exhibited karyokinetic figures showing metaphase- or anaphase-chromosomal configurations (Fig. 5F).

These results show that the pair of asters required for meiotic spindle formation are derived from the polar region of the prophase-arrested oocyte. Therefore, the organizing center(s) (centrosome) for the meiotic spindle probably resides in the pole of the prophase-arrested oocyte.

Discussion

Poles as associated sites of centrosomes

The present study has revealed a pair of asters at the pole of living sea cucumber oocytes during maturation. The occurrence of fibrillar structures (Ohshima, 1921, 1925; Inaba, 1930) and microtubules (Smiley and Cloney,

Table II

Aster formation in GV-enucleate fragments with or without the pole, obtained from prophase-arrested oocytes

Fragments[1]			No. of asters[2]		
Transection	Pole	No.	2	1	Undetected
A	+	23	22 (12)	0	1
B	+	21	21 (21)	0	0
C	+	14	14 (8)	0	0
D	−	15	0	0	15

[1] Prophase-arrested oocytes were transected in various directions (Fig. 4), and GV-enucleate fragments either containing the pole (+) or lacking the pole (−) were treated with 1 mM DTT and observed from about 20 min to about 40 min at room temperature (24–27°C). The fragments lacking the pole were examined further up to about 100 min.

[2] The number of asters detected is indicated. The number in parentheses indicates the fraction of cases in which the site of the pole was recognizable. In these cases, the asters appeared in a cytoplasmic region close to the pole.

1985) at the pole in prophase-arrested oocytes suggests that the asters may pre-exist in some form in immature oocytes. And Oka (1940) reported a fibrillar structure even in growing oocytes. The present study demonstrates that the organizing centers of the meiotic spindle are derived from the pole in prophase-arrested oocytes. These findings imply that the fibrillar structure at the pole is a structure
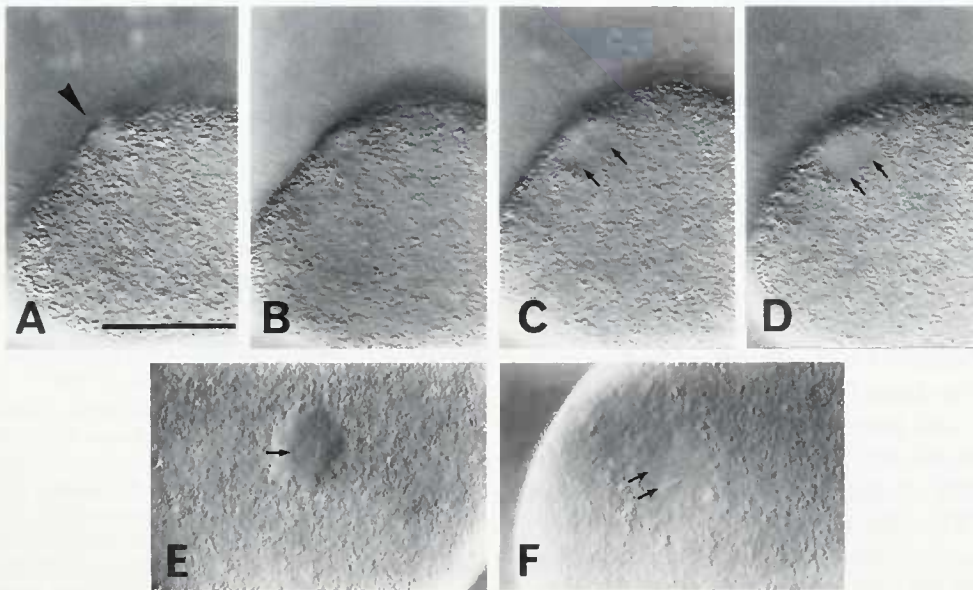
Figure 5. Aster formation in a GV-enucleate fragment with the pole (A–D), and chromosome arrangements in GV-nucleate fragments without (E), and with (F), the pole. The fragment in A–D was obtained from transection A (Fig. 4), and those in E and F were obtained from transections C and D, respectively. A: 9 min. The pole (arrow-head) looks clear. B: 15 min. C: 21 min. A pair of asters (arrows; also in D) form at the pole. D: 30 min. E: 93 min. Chromosomes (arrow) show no karyokinetic figures. F: 65 min. Anaphase chromosomes (arrow). Temperature: 24–27°C. Bar in A: 50 μm (for A–F).

similar to the pre-meiotic aster seen in starfish oocytes (Schroeder, 1985a). I conclude that the pole (*i.e.*, the presumptive animal pole) of the prophase-arrested oocyte of the sea cucumber is the site of the centrosome(s) or microtubule-organizing center(s). With maturation (perhaps, at a time prior to GV breakdown), the centrosome may change to form a pair of asters, and these asters (centrosomes) then function as the organizing centers of the meiotic spindle.

In starfish, the pre-meiotic asters are associated with the cortex at the presumptive animal pole (Schroeder and Otto, 1984, Schroeder, 1985a, b; Picard *et al.*, 1988). A similar microtubule-array containing a microtubule-organizing center occurs in association with the cell surface in GV stage oocytes of sea urchins (Boyle and Ernst, 1989). Hence, the association of the centrosome with the cell surface of the presumptive animal pole where polar bodies later form may be a characteristic common to all echinoderm oocytes.

Poles and meiotic divisions

As can be seen in Figure 1, features specific to oocytes of the sea cucumber, *Holothuria leucospilota*, are the migration of the GV to the pole and the subsequent formation of a meiotic spindle in the polar region. Because the centrosome participating in meiotic spindle formation is initially dissociated from the GV and resides at the pole, a direct outcome of the GV migration is 'restoration' of the spatial association of the centrosome(s) with the nucleus to form the meiotic spindle. The pole, embracing the centrosome (microtubule-organizing center), microtubules, and the cortex, may participate in the migration of the GV to the pole.

Nuclear migration, or spindle migration, and subsequent attachment of the spindle pole to the cortex results in unequal divisions: *e.g.*, polar body formation in surf clam oocytes (Dan and Ito, 1984; Dan and Inoué, 1987), *Chaetopterus* oocytes (Hamaguchi *et al.*, 1983) and *Crepidula* oocytes (Conklin, 1917); micromere formation in sea urchin embryos (Dan, 1979, 1984; Dan *et al.*, 1983; Schroeder, 1987); and ganglion cell formation in neuroblasts of grasshoppers (Kawamura, 1977). The present study of the meiotic divisions of sea cucumber oocytes has revealed that a meiotic spindle eventually "attaches" to a definite site—the pole—to form the polar bodies. Thus, the cortex of the pole is a site specialized for interacting with the spindle-pole aster, and for anchoring the microtubule-organizing center or the asters originating from it. The cortex or cell surface of the pole may contain local factors responsible for binding with asters or the organizing center.

Polar protrusion and clear spot as markers of the animal-vegetal axis in sea cucumber oocytes

The presence of the "clear spot," a special cytoplasmic islet, in the cytoplasm near the cell surface opposite the presumptive animal pole (Maruyama, 1981) could be traced back to prophase-arrested oocytes. With maturation, the clear spot exhibits a rather strong birefringence. Further studies are needed to define its significance in development. In any event, there are now two visible structures, the polar protrusion and clear spot, both serving as markers for the main axis (animal-vegetal axis) of the oocyte. They could be useful for analyzing localized morphogenetic determinants, as has been done in eggs and embryos of sea urchins and starfish (Maruyama *et al.*, 1985; Maruyama and Shinoda, 1990).

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